Histol Histopathol (2015) 30: 1455-1464

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Oligodendroglial markers in the cuprizone model of CNS de- and remyelination

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Summary. Oligodendrocytes are the myelinating cells of the central nervous system. Since many studies of demyelinating diseases focus their research on this cell type, there is growing interest for obtaining reliable markers that can specifically recognize oligodendroglia. Established markers are the myelin-associated neurite outgrowth inhibitor (NogoA), the transcription factor Olig2, and the antibody CC-1, the latter being directed against the protein adenomatous polyposis coli (APC). Unfortunately, it has been discussed whether APC and Olig2 could recognize astrocytes under pathological conditions as well. Hence, we performed immunohistochemical studies using the oligodendroglial markers NogoA, APC, and Olig2 in a murine model of cuprizone induced demyelination. We have found that APC co-localizes with NogoA and does not co-localize with the astrocytic marker GFAP. Olig2 shows co-localization with APC but there is also a small population of Olig2/GFAP double positive cells. Some Olig2/GFAP double positive cells are found in the corpus callosum in a narrow time window in which oligodendrocyte precursor cells proliferate in this model. In other brain regions including the cerebral cortex and hippocampus and in all regions in untreated control mice double positive Olig2/GFAP cells do not occur. In conclusion, our results underline that APC and NogoA are reliable markers for detection of mature oligodendrocytes. Olig2 is a suitable marker to stain cells of oligodendroglial origin but could be combined with GFAP to exclude the GFAP positive population of cells from the quantification of oligodendroglia.

Key words: Oligodendrocytes, Olig2, CC-1, APC, NogoA

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DOI: 10.14670/HH-11-640

Introduction

Oligodendrocytes, as one of the major glial cells in the central nervous system (CNS) generate myelin and maintain the integrity of myelin sheaths by providing trophic factors to axons (Butt et al., 2014). Since myelin is responsible for axonal survival and allows rapid saltatory nerve conduction, disruption of myelin sheaths leads to axonal degeneration and functional deficits (Fields, 2014). CNS demyelination is usually the result of oligodendrocyte damage, which can be caused by metabolic/genetic abnormalities (leukodystrophies) or by inflammatory conditions found in autoimmune diseases such as multiple sclerosis (MS) (Franklin and Kotter, 2008; Stangel, 2012). Remyelination is the natural repair mechanism of myelin damage and can be a highly efficient process. During these pathological processes OPC proliferate, migrate to the lesions, and differentiate into new myelin producing oligodendrocytes (Franklin et al., 2008). The generation of new oligodendrocytes and new myelin formation were proposed to protect against progressive axonal loss and thus long-term disability in MS patients (Keirstead and Blakemore, 1999). For this

reason, oligodendrocytes are in the focus of many research studies that aim to promote remyelination.

General methods to detect myelination and oligodendrocytes in the CNS are immunohistochemical stainings in human CNS tissue from biopsies (Metz et al., 2014) and rodent CNS material (Skripuletz et al., 2011a). For this reason, specific cell markers that allow the unambiguous identification of oligodendrocytes at a specific differentiation state are needed. To date, several surface markers have been established. For instance, oligodendroglial cells can be identified by the myelin-associated neurite outgrowth inhibitor (NogoA) (Kuhlmann et al., 2007), the antibody CC-1 that recognizes the protein adenomatous polyposis coli (APC) (Bhat et al., 1996; Brakeman et al., 1999; Kitada and Rowitch, 2006), and the transcription factor Olig2 (Lu et al., 2000). Unfortunately, there is some controversy regarding the expression specificity of the markers APC and Olig2 given that it has been discussed that these markers might stain astrocytes as well (Cai et al., 2007; Marumo et al., 2013).

The present study aimed to clarify the validity for the use of the markers APC and Olig2 to identify oligodendrocytes in paraffin embedded murine CNS tissue under normal and pathological conditions during de- and remyelination. To induce CNS demyelination we employed the toxic cuprizone mouse model of de- and remyelination (Kipp et al., 2009; Skripuletz et al., 2011a). The cuprizone model is widely accepted to study de- and remyelination processes in the CNS (Gudi et al., 2014).

Material and methods

Experimental design

The objective of the study was to determine the reliability of the markers APC and Olig2 for the identification of oligodendroglial cells. Analyses were performed in untreated murine CNS tissue and under de- and remyelinating conditions in the cuprizone mouse model of de- and remyelination, in which the induction of demyelination was achieved by cuprizone feeding (Skripuletz et al., 2011a).

Animals

C57BL/6 male mice aged 8-10 weeks were purchased from Charles River (Sulzfeld, Germany). Animals underwent routine cage maintenance once a week and were microbiologically monitored according to the Federation of European Laboratory Animal Science Association's recommendations (Rehbinder et al., 1996). Water and food were available *ad libitum*. All procedures involving animals were performed in compliance with the international guidelines on animal care in experimentation and approved by the review board for the care of animal subjects of the district government (Lower Saxony, Germany).

Induction of demyelination

Demyelination was induced with a diet containing 0.2% cuprizone (biscyclohexanone oxaldihydrazone, Sigma-Aldrich, Germany) mixed into a ground standard rodent chow for 5 weeks (Skripuletz et al., 2013). Thereafter, mice were put on normal chow to allow remyelination. At different time points (0 (untreated controls), 3, 4, 5, and 6 weeks) mice were perfused with 4% paraformaldehyde in phosphate buffer via the left cardiac ventricle as previously described (Skripuletz et al., 2013). Six animals per group were analyzed. The brains were dissected, post fixed in 4% paraformaldehyde and paraffin embedded.

Immunohistochemistry

For immunohistochemistry, brains were sliced in 7 μm serial paraffin sections and dried at 37°C overnight. Brain sections between bregma -1.34 mm and -1.94 mm were used for staining procedures (Gudi et al., 2009; Skripuletz et al., 2010). Paraffin embedded sections were de-waxed, rehydrated, and microwaved for 5 min in 10 mM citrate buffer (pH 6.0). Sections were quenched with H_2O_2 and blocked for 1 h in PBS containing 3% normal goat serum and 0.1% Triton X-100. Afterwards, the primary antibody was added for overnight incubation. The following primary antibodies were used: for myelin proteolipid protein (PLP) (1:500, mouse monoclonal immunoglobulin G2a, clone plpc1, Serotec), and cyclic nucleotide 3' phosphodiesterase (CNPase) (mouse immunoglobulin, 1:200, Millipore), for oligodendroglial cells NogoA (1:750, rabbit polyclonal immunoglobulin G, Millipore), CC-1 (APC) (1:200, mouse monoclonal immunoglobulin G2b, clone CC-1, Calbiochem), Olig2 (1:500, rabbit polyclonal immunoglobulin G, Millipore), and CNPase (1:200, mouse monoclonal immunoglobulin G1, clone MAB326R, Millipore), for astrocytes glial fibrillary acidic protein (GFAP) (1:200, rabbit polyclonal immunoglobulin G, DakoCytomation), for activated microglia Mac3 (1:500, rat immunoglobulin G1, clone M3/84, BD Pharmingen).

After washing sections were further incubated with the biotinylated anti-mouse IgG (H+L) secondary antibody (1:500, Vector Laboratories) for 1 h followed by peroxidase-coupled avidin–biotin complex (ABC Kit, Vector Laboratories) for myelin staining. Reactivity was visualized with diamino-3, 3'benzidine (DAB, Vector Laboratories).

For immunofluorescence double stainings treatment with H_2O_2 was omitted, and blocking was performed with PBS containing 10% normal goat serum and 0.1% Triton X-100. The following pairs of primary antibodies were used: NogoA/GFAP, NogoA/Mac3, APC/NogoA, APC/GFAP, APC/Mac3, APC/Olig2, APC/CNPase, Olig2/GFAP, Olig2/Mac3. Sections were then washed with PBS and incubated for 1 h with the appropriate secondary antibodies. The following secondary

antibodies were used: anti-mouse immunoglobulin G1 Alexa-488 conjugated (1:500, Invitrogen), anti-mouse immunoglobulin G2b Alexa-488 conjugated (1:500, Invitrogen), anti-mouse immunoglobulin G2b Alexa-555 conjugated (1:500, Invitrogen), anti-rabbit immunoglobulin G (H+L) Alexa-488 conjugated (1:500, Invitrogen), anti-rabbit immunoglobulin G (H+L) Alexa-555 conjugated (1:500, Invitrogen), and anti-rat immunoglobulin G (H+L) Alexa-488 conjugated (1:500, Invitrogen). Slides were mounted with Mowiol (Calbiochem) containing 4'6-diamidino-2-phenylindole (DAPI; Invitrogen).

Analysis of brain sections and quantification of glial cells

Tissue sections were analyzed for immunopositive cells in white and grey matter brain regions (corpus callosum, cerebral cortex, and hippocampal areas CA3 as well as dentate gyrus) at a magnification of 200 (Olympus BX61) (Skripuletz et al., 2008; Koutsoudaki et al., 2009). In addition, quantification of immunopositive glial cells was performed in the white matter tract corpus callosum (median part). The numbers of counted cells are expressed as number of cells per mm^2.

Statistical analysis

Statistical analysis was performed using analysis of variance (ANOVA) followed by the Fisher-protected least-significant difference test for post hoc comparison if appropriate. All data are given as arithmetic means ± standard error of the mean (SEM).

Results

Cuprizone induced demyelination

To demonstrate that mice show myelin loss, brain sections were immunohistochemically stained for the myelin protein PLP. Slight demyelination occurred after 3 weeks of cuprizone feeding in the corpus callosum, cerebral cortex, and hippocampus. After 5 weeks of

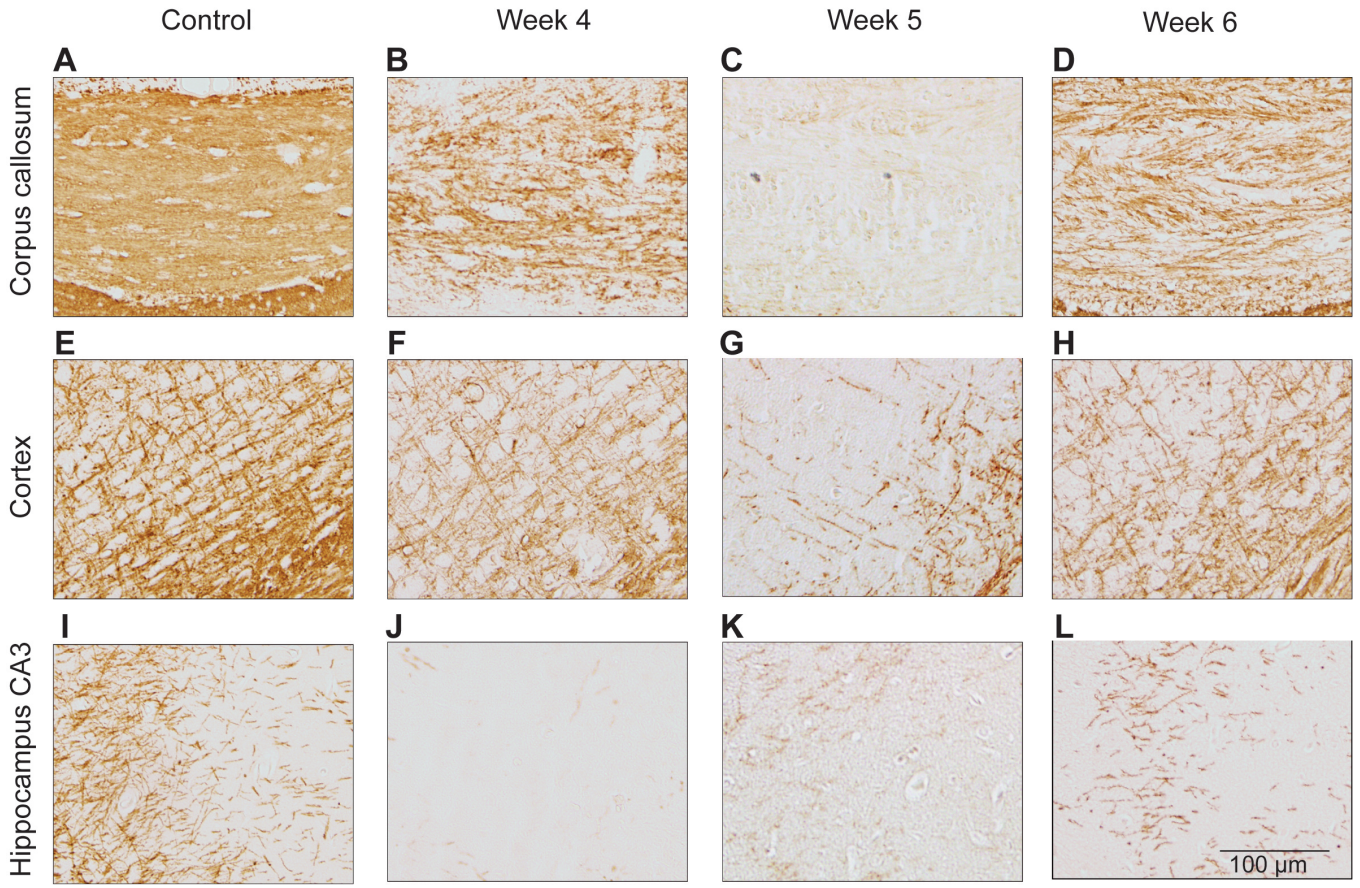

Fig. 1. Immunohistochemical analysis of the myelin marker proteolipid protein (PLP) was performed to determine myelination in the corpus callosum **(A-D)**, cerebral cortex **(E-H)**, and hippocampus **(I-L)** of C57Bl/6 mice during de- and remyelination. **A, E, and I** show control brain sections. **B, F, and J** illustrate severe demyelination after 4 weeks of cuprizone feeding while **C, G, and K** show nearly complete demyelination at week 5 of cuprizone feeding in all regions analyzed. **D, H, and L** show myelin protein re-expression after one week of cuprizone withdrawal (week 6) in the corpus callosum, cerebral cortex, and the hippocampal area CA3.

treatment severe loss of myelin was visible in all brain regions analyzed (Fig. 1). As consequence of cuprizone withdrawal, remyelination occurred at week 6 as shown by the re-expression of the myelin protein PLP (Fig. 1).

The oligodendroglial markers NogoA and APC are not expressed in astrocytes and microglia

Cuprizone feeding is highly toxic for mature oligodendrocytes (Kuhlmann et al., 2007; Benardais et al., 2013). As expected, during cuprizone feeding NogoA$^+$ and APC$^+$ oligodendrocytes disappeared already after 3 weeks of treatment and they were almost completely lost after 4 weeks in all brain regions analyzed. Thereafter, mature oligodendrocytes, as detected by NogoA and APC, re-appeared and new cells could be detected at week 5 in all brain regions (Fig. 2A shows results from the quantification of oligodendrocytes in the corpus callosum).

We further analyzed the expression of NogoA and APC in other glial cell types. For this purpose, immunofluorescence double stainings of NogoA and APC were performed with the astrocytic marker GFAP and the microglial marker Mac3. We found that APC did not co-localize with the astrocytic marker GFAP neither in the corpus callosum nor in the cerebral cortex or hippocampus at all time points (Fig. 3). Similar results were obtained from double stainings with NogoA and GFAP (data not shown). Furthermore, neither NogoA nor APC was expressed in Mac3 positive microglia (data not shown). Thus, our results show that the oligodendroglial markers NogoA and APC neither stain astrocytes nor microglia in untreated control mice and during cuprizone induced demyelination and the subsequent remyelination.

Double stainings for NogoA and APC were performed to investigate co-localization of both markers. We found co-localization of both markers in untreated control mice in all brain regions analyzed. In addition, NogoA and APC co-localized under pathological conditions during de- and remyelination (Fig. 2B shows co-localization of both markers at the time point week 6 in the corpus callosum, cerebral cortex, and the hippocampal areas CA3 and dentate gyrus) except for the time point week 5 in the corpus callosum. At week 5, few cells (4%) expressed the marker APC only in the corpus callosum. As described above, co-localization of APC with the astrocyte marker GFAP or the microglial marker Mac3 was not found. Double stainings with the myelin protein CNPase were performed and revealed co-localization of APC and CNPase (Fig. 2C) confirming that APC positive cells are of oligodendroglial origin.

Olig2 stains oligodendroglial cells and co-localizes in a small population of GFAP positive cells

Olig2 is widely used to detect the complete lineage of oligodendroglial cells including OPC and mature oligodendrocytes (Lu et al., 2000). In the present study we performed double immunofluorescence stainings with the markers Olig2 and APC and we did not observe single positive APC cells. Co-localization of the marker APC with the marker Olig2 was found in untreated control mice and during cuprizone induced demyelination and the subsequent remyelination in all brain regions and all time points analyzed (Fig. 4 shows co-localization in several brain regions). Thus, our results confirm that Olig2 is not only expressed in immature oligodendrocytes, called OPC, but also stains mature APC positive oligodendrocytes.

Co-expression of both markers was observed overall, but also single positive Olig2 cells were detected. In figure 4A, the loss of APC$^+$ and Olig2$^+$ oligodendrocytes is shown in the corpus callosum as consequence of cuprizone feeding. At weeks 3 and 4 increasing numbers of single positive Olig2 cells were observed (Fig. 4B). Thereafter, single positive Olig2 cell numbers decreased while numbers of double positive APC/Olig2 cells increased as a consequence of differentiation of OPC into mature oligodendrocytes.

Further analyses were performed to investigate whether this single positive population of Olig2$^+$ cells might be astrocytes or microglia. Our results show that the Olig2 marker did not co-localize with Mac3$^+$ activated microglia (data not shown). However, Olig2 was observed in the cell nucleus of a small number of GFAP positive cells in specific weeks during cuprizone induced demyelination and only in the corpus callosum (Fig. 5). Olig2 did not occur in GFAP$^+$ cells in all brain regions of untreated mice and in the cerebral cortex and hippocampus during de- and remyelination.

Discussion

Oligodendrocytes are the subject of increasing interest and attention within demyelinating disorders such as multiple sclerosis and the experimental animal models used to study these processes. New aspects of underlying pathomechanisms and the important role of OPC and oligodendrocytes in de- and remyelination processes are being discovered continuously. In this context, reliable markers for the detection of oligodendroglial cells are required. NogoA, APC, and Olig2 are widely used to stain oligodendroglia, but it was suggested that in some pathological conditions APC and Olig2 might be expressed by astrocytes as well (Leroy et al., 2001; Cai et al., 2007).

In the present study, we confirmed that APC serves as a reliable marker for the detection of mature oligodendrocytes. First, we were able to demonstrate that APC does not co-localize with the astrocytic marker GFAP or the microglial marker Mac3 in untreated control mice and in pathological conditions including de- and remyelination. Secondly, APC co-localizes with NogoA, which is another widely used marker for mature oligodendrocytes in normal CNS tissue and under pathological conditions (Kuhlmann et al., 2007). Interestingly, at week 5 we found some single APC

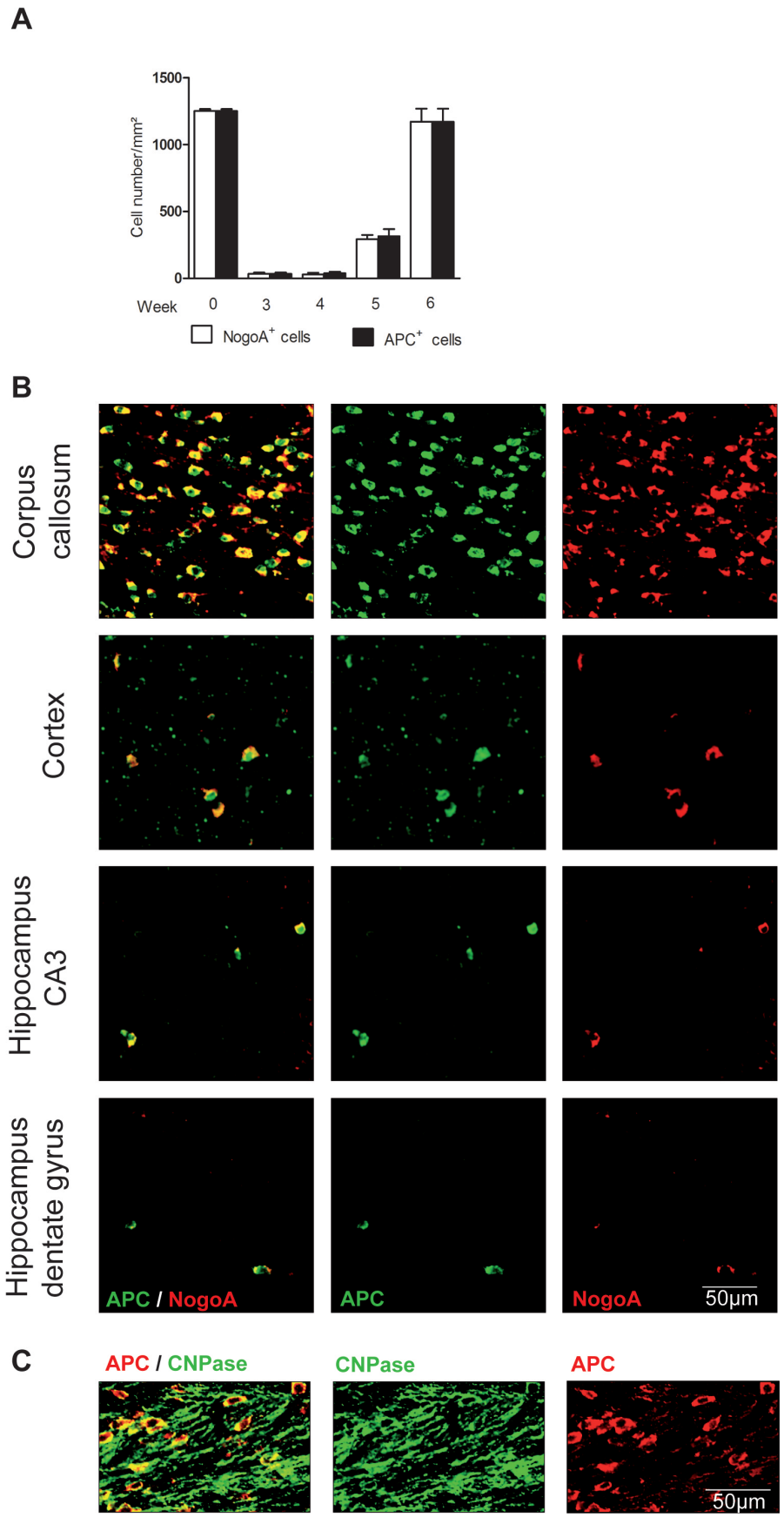

Fig. 2. Immunohistochemical analyses with NogoA and APC were performed in order to analyze oligodendrocytes. NogoA and APC oligodendrocytes decreased in number during demyelination at weeks 3 and 4. At week 5 of treatment new oligodendrocytes appeared in the corpus callosum and reached high numbers at week 6 **(A)**. In **B** representative brain sections show co-localization of APC/NogoA in the corpus callosum, cerebral cortex, and hippocampal areas CA3 and dentate gyrus in control animals and during remyelination at the time point week 6. In **C** double immunofluorescence stainings show co-localization of the oligodendroglial marker APC and the myelin protein CNPase in the corpus callosum during remyelination. Each bar represents the mean ± SEM.

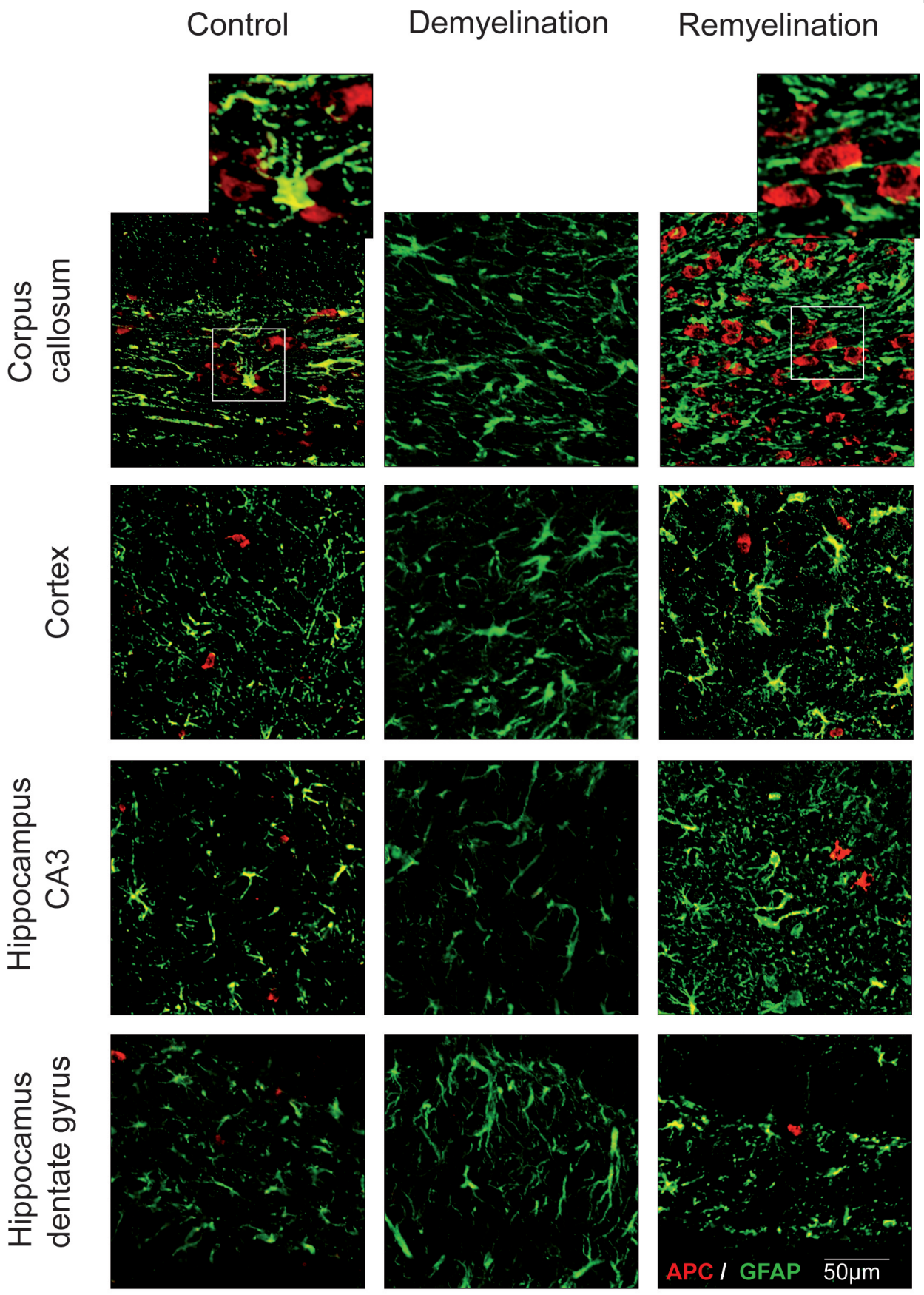

Fig. 3. Double immunofluorescence stainings with APC and GFAP in murine brain sections in untreated mice and during cuprizone induced de- and remyelination. Representative brain sections are shown in the corpus callosum, cerebral cortex, and hippocampal areas CA3 and dentate gyrus in control animals and during demyelination (week 4) and remyelination (week 6). Co-localization of APC and GFAP was not found.

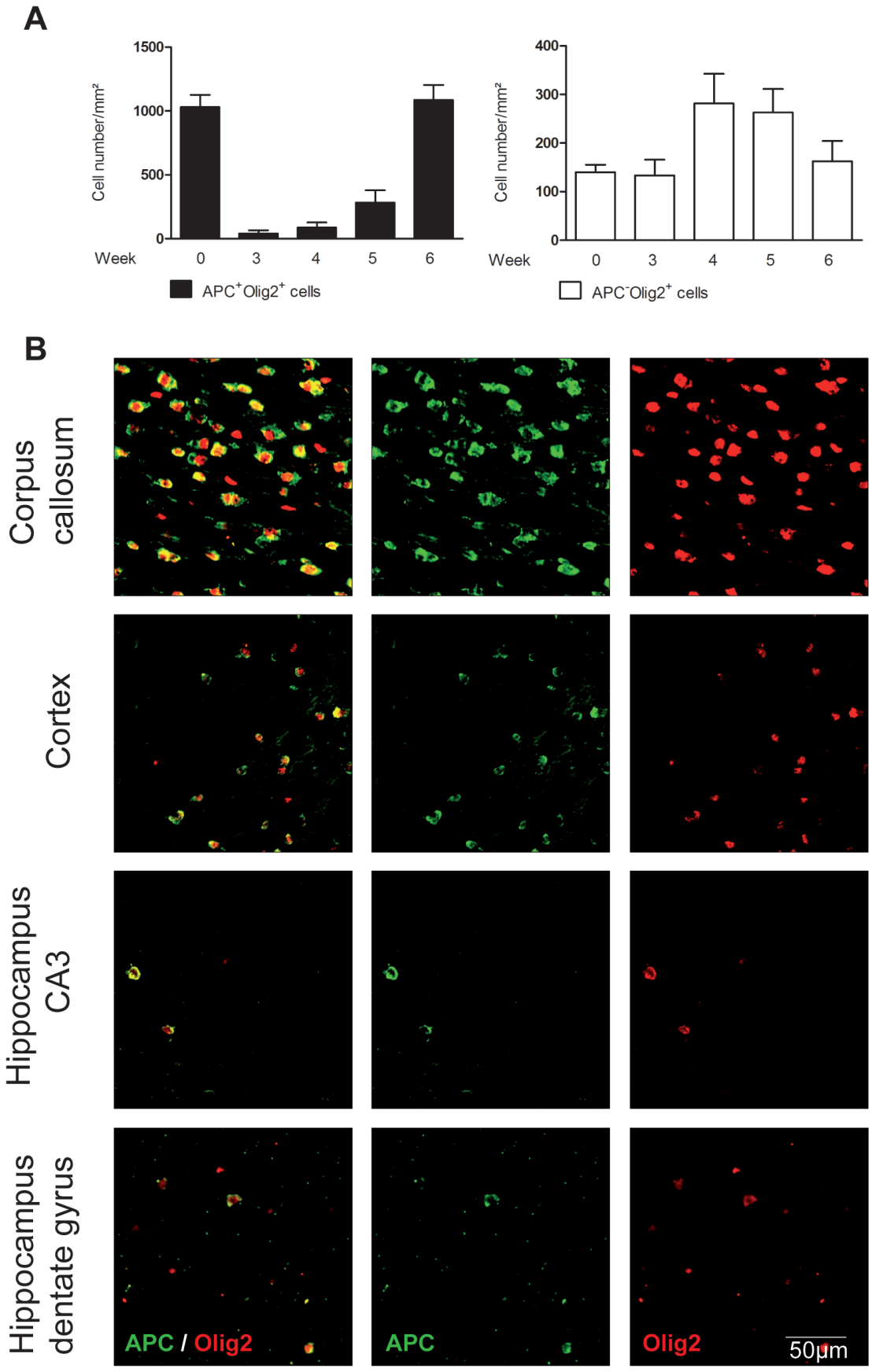

Fig. 4. Double immunofluorescence staining with Olig2 and APC. In **A** results show loss of APC+ and Olig2+ oligodendrocytes in the corpus callosum during cuprizone induced demyelination. At weeks 3 and 4 increasing numbers of single Olig2+ cells were detected. Thereafter, the number of single Olig2+ cells decreased, while the number of APC+/Olig2+ cells increased as a consequence of differentiation of OPC into mature oligodendrocytes. In **B** representative brain sections show co-localization of both markers during remyelination (week 6) in all studied brain regions: corpus callosum, cortex, hippocampus CA3 and dentate gyrus. In addition, a small population of single Olig2+ cells was found in all studied regions. Each bar represents the mean ± SEM.

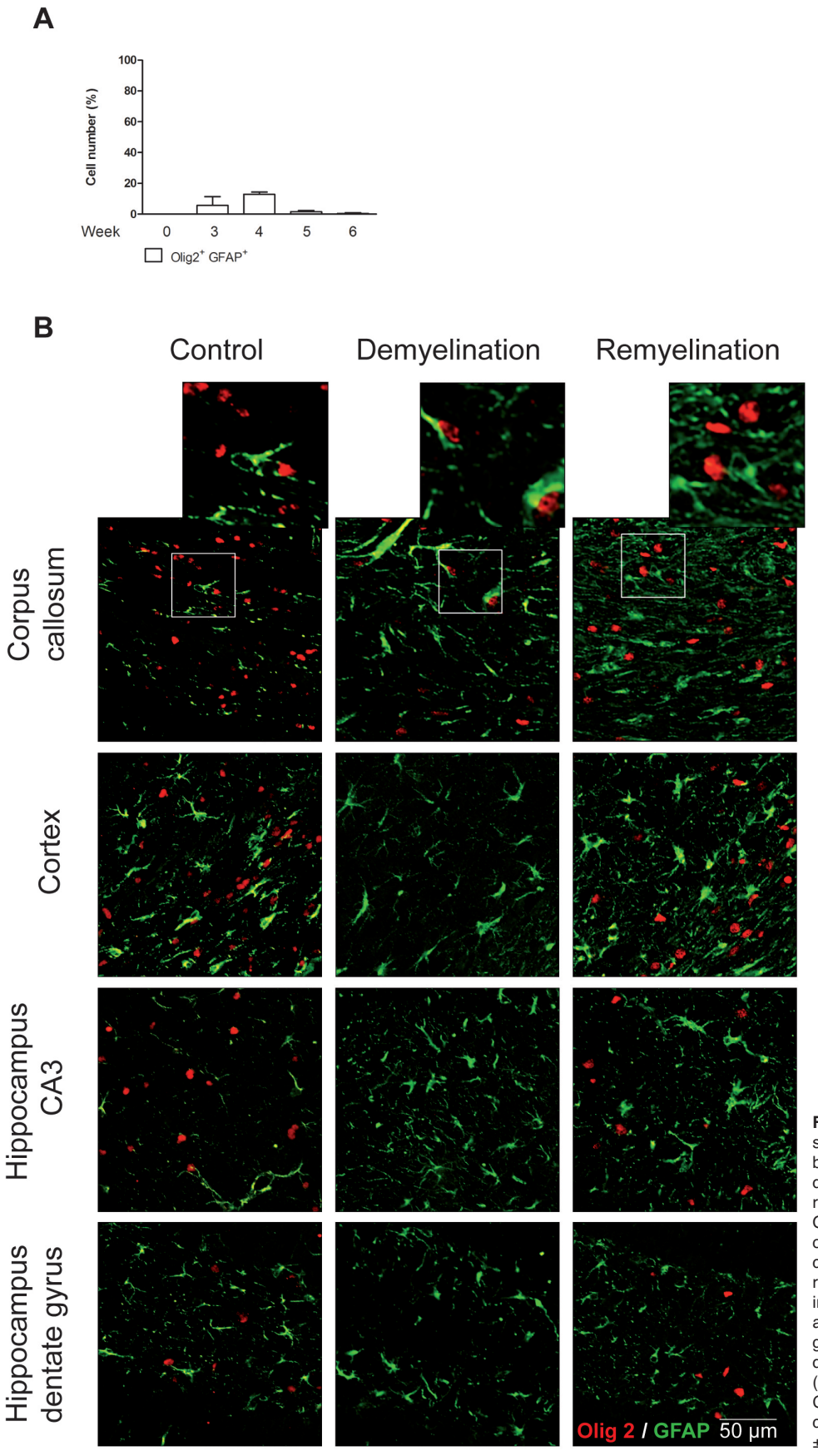

Fig. 5. Double immunofluorescence stainings with Olig2 and GFAP in murine brain sections in untreated mice and during cuprizone induced de- and remyelination. In **A** results show that Olig2 is expressed in a small population of GFAP positive cells in the corpus callosum during demyelination. In **B** representative brain sections are shown in the corpus callosum, cerebral cortex, and hippocampal areas CA3 and dentate gyrus in control animals and during demyelination (week 4) and remyelination (week 6). At week 4, co-localization of GFAP and Olig2 is shown in the corpus callosum. Each bar represents the mean ± SEM.

positive cells in the corpus callosum, but not in the grey matter areas of the cerebral cortex and hippocampus. Double staining with the marker Olig2 showed that these cells are of oligodendroglial lineage since single positive APC cells were not found. Moreover, we found co-localization of APC and the myelin protein CNPase confirming that these cells are of oligodendroglial origin. Newly generated oligodendrocytes appear at this specific time point in the corpus callosum and thus, on the basis of our findings we conclude that APC is expressed earlier in oligodendrocytes than NogoA, which is in accordance with previous studies (Lang et al., 2013). APC was first described as a gut tumor suppressor protein and mutations of the APC gene have been associated with familial polyposis coli and development of bowel neoplasm by regulating the wnt / ß-catenin signaling pathway. However, the interest of this protein was readdressed towards its role in the CNS as high amounts of APC mRNA were observed in the brain tissue (Bhat et al., 1994). Immunostainings using the CC-1 monoclonal antibody against APC were found to specifically stain oligodendrocytes (Bhat et al., 1996). In contrast, antibodies of different clonal origin targeting APC exhibited different immunostaining and expression patterns (Brakeman et al., 1999). Recent works underline that it plays an important role in the differentiation of oligodendrocytes (Lang et al., 2013). Still, the role of APC in the oligodendroglial lineage is poorly understood.

With regard to the marker Olig2, it is well known that Olig2 is expressed in the complete oligodendroglial lineage including OPC and mature oligodendrocytes (Takebayashi et al., 2000). In our study, we confirmed that Olig2 is not only expressed in OPC, but also in mature oligodendrocytes. Immunohistochemical double staining showed co-localization of the markers Olig2 and APC in both untreated control mice and in mice during de- and remyelination in the white and grey matter areas of the brain. Single positive APC cells were not found, indicating that Olig2 is expressed by mature oligodendrocytes. Consequently, the occurrence of single Olig2 positive cells might be attributed to OPCs. Additionally, we have shown co-expression of Olig2 with the astrocytic marker GFAP in a small cell population (13% at week 4) during cuprizone induced demyelination. Double positive cells were found only in the corpus callosum in a narrow time window that is known to be the peak of OPC regeneration in the cuprizone model (Gudi et al., 2009; Skripuletz et al., 2011b). We did not find any double positive Olig2/GFAP cells in other brain regions, including the cerebral cortex and hippocampus and in all regions in untreated control mice. It is controversially discussed if these cells present a transient population of OPC or rather astrocytes. Olig2 is a transcription factor that is important for oligodendrocyte and motoneuron fate specification (Takebayashi et al., 2000; Mizuguchi et al., 2001). Since it is also a characteristic marker for immature glial progenitor cells (Magnus et al., 2007), Olig2 has been assigned a role in astrocytes (Rowitch, 2004). It has been shown that astrocytes are able to express Olig2 under pathological conditions (Chen et al., 2008). After cortical injury Olig2 was upregulated in reactive astrocytes while Olig2 ablation decreased the proliferation of reactive astrocytes. Olig2 is transiently expressed in astrocytes during early postnatal development (Cai et al., 2007), but because it represses the transcription of GFAP, its cytoplasmatic translocation is required for astrocytic differentiation (Fukuda et al., 2004; Setoguchi and Kondo, 2004). In our work, we found Olig2 in the nucleus and thus we speculate that these cells are rather reactive OPC. Furthermore, $GFAP^+$ type B astrocytes in the subventricular zone are regarded as oligodendrocyte progenitors contributing to remyelination (Mecha et al., 2013). Other studies have indicated that GFAP is expressed by a population of OPC which give rise to Schwann cells in the CNS (Blakemore, 2005).

In conclusion, we determined that APC and NogoA are reliable markers to detect mature oligodendrocytes in the toxic cuprizone model of demyelination. We confirmed that mature oligodendrocytes also express the Olig2 marker. Olig2 was found in a small population of GFAP positive cells only in the corpus callosum under pathological conditions in a narrow time window. Olig2 did not colocalize with GFAP under resting conditions and in other brain regions during de- and remyelination, indicating that it is a suitable marker to stain cells of oligodendroglial origin.

Acknowledgements. We thank I. Cierpka-Leja, S. Lang, and A. Niesel for excellent technical assistance. This work is part of L. Salinas Tejedor's PhD thesis. Thomas Skripuletz is supported by the Genzyme Neuroimmunology Fellowship. Thomas Skripuletz and Martin Stangel are supported by the German Research Foundation (DFG, FOR 1103, TP1b).

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Accepted June 24, 2015